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Applicant: Stefan Kochanek et al. Art Unit:  
Serial No.: 10/079,609 Examiner:  
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Title: PIGMENT EPITHELIAL CELL OF THE EYE, ITS PRODUCTION AND  
USE IN THERAPY OF AN EYE OR CNS DISEASE  
Assistant Commissioner For Patents  
Washington, D.C. 20231

SUBMISSION OF VERIFIED ENGLISH TRANSLATION

To complete the record in this case, Applicants submit the accompanying English translation of the provisional application relied upon for priority in the above-referenced case. In particular, the above-referenced application claims the benefit of this provisional application, U.S.S.N. 60/270,746, filed February 22, 2001. Applicants hereby verify that this English translation is true and accurate as required by 37 C.F.R. § 1.52(d).

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Respectfully submitted,

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I, Elisabeth Ann LUCAS,

Director of RWS Group plc, of Europa House, Marsham Way, Gerrards Cross,  
Buckinghamshire, England declare;

1. That I am a citizen of the United Kingdom of Great Britain and Northern Ireland.
2. That the translator responsible for the attached translation is well acquainted with the German and English languages.
3. That the attached is, to the best of RWS Group plc knowledge and belief, a true translation into the English language of the specification in German filed with the application for a patent in the U.S.A. on  
under the number
4. That I believe that all statements made herein of my own knowledge are true and that all statements made on information and belief are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing thereon.

For and on behalf of RWS Group plc

The 21st day of December 2001

February 22, 2001

Dr. med. Stefan Kochanek

K35245US BÖ/ATe/plö

Dr. rer. nat. Ulrich Schraermeyer

5 **Pigment epithelial cell of the eye, its production and  
use in therapy of an eye or nerve disease**

The present invention relates to a pigment epithelial  
cell of the eye comprising vector DNA of an adenoviral  
10 vector with large DNA capacity, to the improved  
isolation and cultivation of these cells and to methods  
for the production and the use in therapy of an eye or  
nerve disease.

15 The five primary senses of touch, sight, hearing, taste  
and smell serve to pick up information from the  
surroundings. About 75% of our perceptions involve the  
sense of sight. This high percentage makes it clear  
that sight is of predominant importance in our daily  
20 life. Consequently, a weakening of our vision  
represents a great intrusion into everyday life.

The eye consists of a compound lens system which  
produces an image, which is inverted and reduced in  
25 size, of the surroundings on the retina. The dioptic  
apparatus consists of the transparent cornea, the iris  
forming the pupil, the lens and the vitreous body, a  
gelatinous, transparent mass inside the eyeball between  
lens and retina. Fig. 1 shows a schematic horizontal  
30 section through the eye. The covering of the eyeball  
consists of 3 layers: the sclera, the choroid and the  
retina. The retina in turn consists of an outer layer,  
the retinal pigment epithelium (RPE), and an inner  
layer, the neurosensory retina.

35 The annular iris separates the anterior from the  
posterior chamber of the eye and forms the anterior  
part of the uvea. From anterior to posterior it  
consists of a collagen-rich extracellular matrix, the

iris stroma (it contains melanocytes, fibrocytes, nerves and blood vessels) and the iris pigment epithelium (IPE).

5 The iris pigment epithelium is in two layers and consists of an anterior and a posterior pigment epithelial cell layer (Freddo TF (1996) Ultrastructure of the iris. Microsc Res Tech 33: 369-389). The cells of the posterior iris pigment epithelium are connected  
10 by tight junctions. The anterior pigment epithelium has in addition smooth muscle cells (except in the region of the sphincter) which contribute to dilatation of the iris (Freddo 1996, supra). The iris pigment epithelium has the same embryological origin as the retinal  
15 pigment epithelium. It is possible to obtain about  $2.3 \times 10^5$  IPE cells from a human iris, 90% of which survive in cell culture (Hu DN, Ritch R, McCormick SA, Pelton-Henrion K (1992) Isolation and cultivation of human iris pigment epithelium. Invest Ophthalmol Vis Sci 33:  
20 2443-2453). IPE cells are highly pigmented and contain much eumelanin. Melanin has the following protective functions. It is able to bind divalent iron ions ( $\text{Fe}^{2+}$ ) and other toxic substances (e.g.  $\text{Ca}^{2+}$ ) and thus remove them from the cytoplasm of the cell (Hill HZ (1992) The  
25 function of melanin or six blind people examine an elephant. Bioessays 14: 49-56). Melanin is additionally able to convert  $\text{Fe}^{2+}$  into less toxic  $\text{Fe}^{3+}$  by redox reactions. On the other hand, the melanin synthesis precursors dihydroxyindole (DHI) and dihydroxyindole-  
30 carboxylic acid (DHIA) have a very strong antioxidant effect which is stronger than that of alpha-tocopherol (Memoli S, Napolitano A, d'Ischia M, Misuraca G, Palumbo A, Prota G (1997) Diffusible melanin-related metabolites are potent inhibitors of lipid  
35 peroxidation. Biochim Biophys Acta 1346: 61-68). Melanin is able to eliminate toxic oxygen free radicals produced in the eye by the high partial pressure of oxygen in combination with exposure to light. Elements important for the normal function of the retina, such

as, for example, zinc, are moreover stored by melanin with great efficiency. Zinc, as a cofactor for, for example, antioxidative enzymes (superoxide dismutase) or connective tissue-degrading enzymes (metalloproteinases), has several important functions in the eye and in the central nervous system (CNS).

The pigment epithelium plays an import part in metabolism and in absorption of light in the eye. It is additionally responsible for the outer blood-retina barrier and for disposing of rejected photoreceptor cells. Consequently it forms an interesting target for the gene therapeutic treatment of eye diseases.

To date, a few experiments on the genetic modification of pigment epithelial cells have been described, but these provided unsatisfactory results in terms of the duration and stability of expression.

In a study with laboratory mice, a first generation adenoviral vector which expressed the E.coli lacZ gene under the control of the CMV promoter was used for gene transfer into the retinal pigment epithelium by subretinal injection. First generation adenoviral vectors (Gilardi et al., FEBS Letters 267, 60-62, 1990; Stratford-Perricaudet et al., Hum. Gene Ther. 1, 241-256, 1990) are characterized by deletions of the E1A and E1B genes. E1A and E1B have transforming and transactivating properties. In some vectors there is also deletion of E3 in order to increase the capacity to take up foreign DNA. Although the gene transfer into the retinal pigment epithelium was efficient and very good expression was observed shortly after injection in the retinal pigment epithelium, the expression was transient. 6 weeks after the injection, only a few lacZ-positive retinal pigment epithelial cells were still observable (Li T, Adamian M, Roof DJ, Berson EL, Dryia TP, Roessler BJ, Davidson BL (1994) In vivo

transfer of a reporter gene to the retina mediated by an adenoviral vector. Invest Ophthalmol Vis Sci: 35, 2543-2549).

5 A further study carried out on laboratory rats with an observation period of 14 days used a first generation adenovirus which expressed the E.coli lacZ gene under the control of the Rous Sarcoma Virus (RSV) promoter. Although the gene transfer into the retinal pigment epithelium was efficient, and very good expression was  
10 observed 7 days after the injection in the retinal pigment epithelium, the expression was reduced one week later (Rakoczy PE, Lai CM, Shen WY, Daw N, Constable IJ (1998) Recombinant adenovirus-mediated gene delivery into the rat retinal pigment epithelium in vivo.  
15 Australian and New Zealand Journal of Ophthalmology 26 (Suppl.): S56-S58).

Another study carried out on 6-week-old RCS rats used a first generation adenoviral vector which expressed the  
20 green fluorescence protein (GFP) gene under the control of the CMV promoter (Anglade E, Csaky KG (1998) Recombinant adenovirus-mediated gene transfer into the adult rat retina. Curr Eye Res 17: 316-321). Although the gene transfer into the retinal pigment epithelium  
25 after subretinal injection was efficient, and 30 to 90% of the retinal pigment epithelium were GFP-positive in the region of the injection site 3 days after the injection, GFP expression was no longer detectable 6 days later (that is 9 days after the injection).

30 Whereas first generation adenoviral vectors were used in the abovementioned examples of gene transfer in the region of the eye, in a further publication there was use of an adenoviral vector which is referred to as  
35 adenovirus minichromosome (EAM) for subretinal injection of mice (Kumar-Singh R, Farber DB (1998) Encapsidated adenovirus mini-chromosome-mediated delivery of genes to the retina: application to the

rescue of photoreceptor degeneration. Hum Mol Genet 7: 1893-1900). This comprises a vector which does not express any viral proteins. The vector expressed the beta unit of cyclic GMP phosphodiesterase (PDE) under the control of the natural PDE promoter. The vector also expressed the E.coli lacZ gene under the control of the CMV promoter. In addition, the vector contained various E.coli plasmid elements (Plasmid backbone, ampicillin resistance gene, E.coli origin of replication). After production, the vector was characterized by pronounced variability of its genome. Monomeric and dimeric structures were observed, the latter in head-to-head, head-to-tail and tail-to-tail orientation. Because of this variability and the presence of plasmid sequences including antibiotic resistance, this vector is unsuitable for therapeutic use. The gene transfer experiments were carried out on rd mice which represent an animal model of retinal degeneration and are characterized by a mutation, which causes the degeneration, in the beta unit of the PDE gene. In this study, expression was detected exclusively in the neuronal portion of the retina but not in the retinal pigment epithelium. Although the neuronal cells are post-mitotic and thus no longer able to divide, expression of the PDE gene was only transient. Various methods (RT-PCR, Western blot analysis and determination of the PDE activity) were used to demonstrate that expression was no longer detectable 4 months after the injection.

To date, only the nonviral transfection reagent Lipofectamine has been used for transfecting IPE cells. In this study, the plasmid pXCN2-bFGF which expresses the rat bFGF cDNA was produced. The plasmid additionally contains a neomycin resistance gene. Cultivated rat IPE cells were transfected with this plasmid. The cells expressed the bFGF cDNA in vitro, and the authors write that degeneration of photoreceptors was delayed by up to 4 weeks in the RCS

rat after subretinal transplantation as cell suspension  
(Tamai M, Yamada K, Takeda N, Tomita H, Abe T, Kojima  
S, Ishiguro I (1997) bFGF transfected iris pigment  
epithelial cells rescue photoreceptor cell degeneration  
5 in RCS rats. In: La Vail M, eds. Degenerative retinal  
diseases. 323-328). However, since, as shown in the  
work mentioned, the same effect, namely delayed  
degeneration of photoreceptors, was observed also in  
rats which had received IPE cells after transfection  
10 with a control plasmid by subretinal injection, and was  
not improved or extended by the transfection, this  
effect was not one which could be attributed to a  
targeted gene transfer but was explicable solely by the  
transplantation of the IPE cells. In addition, bFGF  
15 expression after transplantation was not demonstrated.

The publications mentioned therefore do not disclose an  
expression system for pigment epithelial cells of the  
eye with which long-term stable expression of an  
20 introduced gene can be observed. Long-term stable  
expression of such a gene is, however, necessary for  
the therapy of a large number of hereditary and  
acquired eye diseases. For many applications it is  
precisely the long-term production of therapeutic  
25 proteins which is the crucial factor for achieving a  
therapeutic effect.

It is therefore an object of the present invention to  
provide pigment epithelial cells of the eye which can  
30 be employed in therapy.

The object is achieved by providing a pigment  
epithelial cell of the eye.

35 It has now been found, surprisingly, that a pigment  
epithelial cell of the eye which comprises vector DNA  
of an adenoviral vector with large DNA capacity shows  
long-term stable expression of at least one introduced  
gene and thus can be employed in therapy.



One aspect of the present invention is a pigment epithelial cell of the eye which comprises a vector DNA of an adenoviral vector with large DNA capacity.

5

A pigment epithelial cell of the eye means an epithelial cell of the eye in which pigment, for example melanin, is incorporated. An example of a pigment epithelial cell of the eye is a retinal pigment epithelial cell (RPE) or an iris pigment epithelial cell (IPE).

15 An adenoviral vector of large DNA capacity is understood by the skilled worker to be adenoviruses which comprise no viral coding DNA sequences (Kochanek S, Clemens PR, Mitani K, Chen HH, Chan S, Caskey CT (1996) A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. Proc Natl Acad Sci U.S.A. 93: 5731-5736; 20 Fisher KJ, Choi H, Burda J, Chen SJ, Wilson JM. (1996); Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. Virology 217: 11-22; Kumar-Singh R, Chamberlain JS (1996) Encapsidated 25 adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. Hum Mol Genet 5: 913-921). These adenoviruses contain only the viral ends with inclusion of the inverted terminal repeats (ITRs) and the packaging signal. The 30 capacity to take up foreign DNA is, for example, up to about 37 kb, because the predominant part of the adenoviral genome has been deleted.

35 Adenoviruses are particularly important as expression vectors, especially in the framework of gene therapy. One advantage of adenoviral vectors is the fact that these vectors are able to transduce replicating and nonreplicating cells efficiently *in vitro* and *in vivo*.

Various systems for producing adenoviral vectors of large DNA capacity have been described (Kochanek S (1999) High-capacity adenoviral vectors for gene transfer and somatic gene therapy. Hum Gene Ther 10: 2451-2459). The advantage of these adenoviral vectors with large DNA capacity compared with first and second generation adenoviral vectors is in particular the larger capacity. This makes it possible to introduce one or more genes or expression cassettes into the pigment epithelial cells.

After uptake of the adenoviral vector into the cell, the coat of the vector is normally broken down in endosomes. The remaining vector DNA is then transported into the cell nucleus and usually does not integrate into the cellular genome.

One example of an adenoviral vector with large DNA capacity is a vector which expresses the human alpha1-antitrypsin gene (Schiedner G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C, Graham FL, Beaudet AL, Kochanek S (1998) Genomic DNA transfer with a high capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. Nature Genetics 18: 180-183). Another example is a vector which expresses the dystrophin gene and E.coli lacZ genes (Kochanek S, Clemens PR, Mitani K, Chen HH, Chan S, Caskey CT (1996) A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. Proc Natl Acad Sci U.S.A. 93: 5731-5736). In a preferred embodiment, HC-AdFK7 or HC-AdhCMV.PEDF is used as adenoviral vector with large DNA capacity. HC-AdFK7 is an adenoviral vector with large DNA capacity which expresses the enhanced green fluorescence protein (EGFP) under the control of the human cytomegalievirus promoter. HC-AdhCMV.PEDF is an adenoviral vector with large DNA capacity which expresses the human pigment epithelial cell-derived

factor (PEDF) gene under the control of the  
cytomegalovirus promoter. In this vector, the PEDF  
protein is tagged by attachment (expression as fusion  
protein) of a polyhistidine epitope, so that the  
5 protein can easily be detected by use of an anti-  
polyhistidine antibody.

As shown in the examples, pigment epithelial cells can  
be transduced very efficiently in vitro with an  
10 adenoviral vector of large DNA capacity. As likewise  
shown in the examples, transplantation of these  
genetically modified cells is followed by a long-term  
gene expression which can be detected continuously for  
at least 4 months. It is evident that the  
15 transplantation site is not critical in this  
connection. After transplantation of the genetically  
modified pigment epithelial cells both into the eye in  
the subretinal space and, particularly surprisingly,  
into the CNS in the Corpus striatum led to long-term  
20 gene expression detectable for at least 4 months (eye)  
and at least 2 months (CNS).

Most experiments with these vectors have to date been  
carried out in the liver and in the skeletal muscle.  
25 Although liver gene transfer with a vector expressing  
human alpha1-antitrypsin into baboons (Morral N, O'Neal  
W, Rice K, Leland M, Kaplan J, Piedra PA, Zhou H, Parks  
RJ, Velji R, Aguilar-Cordova E, Wadsworth S, Graham FL,  
Kochanek S, Carey KD, Beaudet AL (1999) Administration  
30 of helper-dependent adenoviral vectors and sequential  
delivery of different vector serotype for long-term  
liver-directed gene transfer in baboons. Proc Natl Acad  
Sci USA 96: 12816-12821) was followed by longer-term  
expression (longer than one year) in two of three  
35 animals, in contrast to the present invention there was  
observed to be a continuous decrease in expression,  
which was still 19% of the initial levels in one of the  
animals after 16 months, and still 8% in the second  
animal after 24 months. In a third animal there was

complete loss of expression within 10 weeks. There was speculation about the reason for the slow decrease in expression in the two animals with prolonged expression. Both growth of the animals and slow cell  
5 division of the hepatocytes were discussed. In the final analysis, the cause of the slow loss of expression is not explained. In the animal in which rapid loss of expression was observed, production of antibodies directed against human alphas-1 antitrypsin  
10 was observed.

A distinct decrease in expression of a LacZ reporter gene after gene transfer into the liver with an adenoviral vector of large DNA capacity within 30 days  
15 after the injection was observed in a further study (Parks RJ, Bramson JL, Wan Y, Addison CL, Graham FL (1999) Effects of stuffer DNA on transgene expression from helper-dependent adenovirus vectors. J Virol 73: 8027-8034).

20 Skeletal muscle is a tissue which, in terms of the natural turnover of the cells, resembles the pigment epithelial cells of the eye. Skeletal muscle cells are postmitotic cells. This means that they are similar to  
25 pigment epithelial cells in no longer dividing. There has been particular experience in gene transfer into the skeletal muscle of laboratory animals using adenoviral vectors of large DNA capacity. The gene transfer in these experiments was effected by direct  
30 injection into the tissue, similar to the present invention. Although expression over a prolonged period was observed after gene transfer using an adenoviral vectors of large DNA capacity which expressed both the dystrophin cDNA and the E.coli LacZ gene, it was  
35 observed that expression once again decreased within 84 days (Chen HH, Mack LM, Kelly R, Ontell M, Kochanek S, Clemens PR (1997) Persistence in muscle of an adenoviral vector that lacks all viral genes. Proc Natl Acad Sci USA 94: 1645-1650). There was complete loss of

expression after 84 days in immunocompetent animals displaying no tolerance to E.coli beta-galactosidase.

5 In contrast to the experiments described in the literature, the stability of expression of genes introduced by gene transfer with an adenoviral vector of large DNA capacity into the pigment epithelial cell was surprisingly high. The advantages of adenoviral vectors of large DNA capacity in the transfection  
10 according to the invention of pigment epithelial cells of the eye compared with known transfection systems are accordingly

- the ensuring of stable gene expression;
- 15 • the possibility of achieving regulated gene expression by use of constitutive, tissue-specific, regulatable promoters and regulatable expression systems;
- the lack of immunogenicity and toxicity of the  
20 vector;
- the high transduction efficiency on use of pigment epithelial cells.

In a preferred embodiment, therefore, the adenoviral  
25 vector comprises a therapeutic nucleic acid, in particular a therapeutic DNA, which does not originate from the adenoviral vector. This might be, for example, a therapeutic gene. A therapeutic gene is understood by the skilled worker to be a gene whose expression  
30 product can be used for the therapy or diagnosis of a disease.

A nucleic acid means a polymer which is cleaved on hydrolysis into sugars, in particular pentoses,  
35 especially ribose and deoxyribose, heterocyclic organic bases, in particular adenine, cytosine, guanine, thymine and uracil, and phosphoric acid. The nucleic acid may be, for example, a DNA or RNA. A therapeutic

nucleic acid is a nucleic acid which itself or the product thereof has a therapeutic effect.

A gene means a linear DNA section which codes for a protein or an RNA. The therapeutic gene which is introduced by gene transfer into the pigment epithelial cell may vary in nature. The choice is determined by the therapeutic aim. For example, a gene which codes for a neurotrophic factors can be used. Examples of neurotrophic factors are the glial cell-derived neurotrophic factor (GDNF) and the pigment epithelial cell-derived factor (PEDF). It is also possible, for example, to use genes which prevent neoangiogenesis. One example is the soluble receptor for the vascular endothelial cell growth factor (VEGF) which is called soluble vascular endothelial cell growth factor receptor-1 (sflt1) (Roeckl W, Hecht D, Sztajer H, Waltenberger J, Yayon A, Weich HA (1998) Differential binding characteristics and cellular inhibition by soluble VEGF receptors 1 and 2. Experimental cell research 241:161-1709. Another example is a dominant-negative VEGF receptor 2 (KDR) (Machein MR, Risau W, Plate KH (1999) Antiangiogenic gene therapy in a rat glioma model using a dominant-negative vascular endothelial growth factor receptor 2. Hum Gene Ther 10: 1117-1128). Further therapeutic genes might be, for example, NGF, BDNF, CNTF, bFGF or neurotrophin 3,4-5.

PEDF has a very strong neurotrophic and neuroprotective effect (King GL, Suzuma K (2000) Pigment-epithelium-derived factor - a key coordinator of retinal neuronal and vascular functions. N Engl J Med 342: 349-351). This factor is produced by RPE under normoxic conditions. Production is stopped during hypoxia. This greatly promotes neovascularization. In age-related macular degeneration (AMD) the damaged RPE cells produce too little PEDF. This produces uncontrolled neoangiogenesis. The central effect of PEDF in the eye is to prevent neogenesis of vessels.

It is therefore possible according to the present invention for a genetically modified pigment epithelial cell to be a pigment epithelial cell which, after  
5 genetic modification with a PEDF-expressing adenoviral vector of large DNA capacity, secretes PEDF. This cell can then, for example, be transplanted into the subretinal space near the macula of patients following surgical removal of neovascularization membranes. The  
10 pigment epithelial cell is thus able on the one hand to replace the removed retinal pigment epithelium, and on the other hand to produce the PEDF factor essential for preventing neovascularization. Vision is stabilized in this way. PEDF is additionally able to protect from  
15 glutamate-mediated neurotoxicity.

It is additionally possible, depending on the cause of the disease, for various therapeutic genes to be expressed, singly or in combination, by the adenoviral  
20 vectors of large DNA capacity.

In another preferred embodiment, the gene is under the control of a viral or nonviral promoter which has constitutive, tissue-specific and/or regulatable  
25 activity.

A promoter which has constitutive activity means a promoter which mediates transcription of the downstream gene in virtually all tissues and virtually  
30 independently of the physiological state of the cell. An example of a promoter which has constitutive activity is the SV40 or the cytomegalovirus promoter.

A tissue-specific promoter means a promoter which  
35 mediates transcription of the downstream gene only in a particular tissue. Use of the tissue-specific promoter allows a protein or a functional RNA to be expressed tissue-specifically in IPE or in RPE cells. An example of such a tissue-specific promoter is the transthyretin

promoter, which has good activity in RPE and in IPE cells.

A regulatable promoter means a promoter which mediates  
5 the transcription of a gene for example depending on  
the metabolic situation in the cell, the concentration  
of a molecule or the temperature. Gene expression can  
be controlled quantitatively and qualitatively by use  
of a regulatable promoter. An example of a regulatable  
10 promoter is a promoter which is activated in the event  
of hypoxia through inclusion of a hypoxia-sensitive  
element (Boast K, Binley K, Iqball S, Price T, Spearman  
H, Kingsman S, Kingsman A, Naylor S (1999)  
Characterization of physiologically regulated vectors  
15 for the treatment of ischemic disease. Hum Gene Ther  
10: 2197-2208).

However, it is also possible to use a regulatable  
expression system which, for example, is induced or  
20 inactivated on administration of a medicament. An  
example of such a system is a tetracycline-dependent  
gene expression system (Freundlieb S, Schirra-Muller C,  
Bujard H (1999) A tetracycline controlled activation/  
repression system with increased potential for gene  
25 transfer into mammalian cells. J Gene Med 1:4-12).

After transduction of the pigment epithelial cell with  
an adenoviral vector with large DNA capacity, the cell  
is able to produce therapeutic proteins or RNAs. The  
30 therapeutic protein is a protein which causes a  
therapeutic effect. An analogous statement applies to a  
therapeutic RNA, for example an antisense RNA or a  
ribosyme. Examples of therapeutic proteins are the  
neurotrophic factors PEDF, GDNF, NGF, BDNF, CNTF, bFGF  
35 or neurotrophin 3,4-5 (Friedman WJ, Black IB, Kaplan DR  
(1998) Distribution of the neurotrophins brain-derived  
neurotrophic factor, neurotrophin-3, and neurotrophin-  
4/5 in the postnatal rat brain: an immunocytochemical  
study. Neuroscience 84: 101-114) and factors with anti-



angiogenetic activity, such as, for example, the soluble VEGF receptor-1 (sflt-1), the dominant-negative VEGFR-2 (KDR), and once again PEDF, which also has an antiangiogenetic activity in addition to its  
5 neurotrophic function (Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W, Bouck NP (1999) Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 285: 245-248).

10 Further examples of therapeutic genes are lysosomal enzymes (Cingle KA, Kalski RS, Bruner WE, O'Brien CM, Erhard P, Wyszynski RE (1996) Age-related changes of glycosidases in human retinal pigment epithelium. Curr Eye Res 115: 433-438) alpha-mannosidase, beta-  
15 galactosidase, N-acetyl-beta-glucosaminidase and N-acetyl-beta-galactosaminidase, and lipase. These enzymes play an important part in the breakdown of visual cell membranes and may be reduced in AMD.

20 Some more examples are genes which code for anti-oxidative enzymes (superoxide dismutase, catalase, peroxidases) because they may likewise be involved in the pathogenesis of AMD (Frank RN, Amin RH, Puklin JE (1999) Antioxidant enzymes in the macular retinal  
25 pigment epithelium of eyes with neovascular age-related macular degeneration, J Ophthalmol 127: 694-709).

Further examples are genes for gene products which are able to increase choroidal blood flow, for example NO  
30 synthases, because reduced choroidal blood flow may be involved in the pathogenesis of AMD (Luksch A, Polak K, Beier C, Polska E, Wolzt M, Dorner GT, Eichler HG, Schmetterer L (2000) Effects of systemic NO synthase inhibition on choroidal and optic nerve head blood flow  
35 in healthy subjects. Invest Ophthalmol Vis Sci 41: 3080-3084).

A further aspect of the present invention is a pigment epithelial cell in a fixed assemblage of cells, called

a cell sheet. To date, only single cell suspensions of autologous IPE cells have always been transplanted during experimental therapy of AMD. The advantage of such cell sheets is that the cells can be sited  
5 distinctly better by the transplantation technique, and that migration of cells away from the site of transplantation is prevented. Pigment epithelial cells in a fixed assemblage of cells are characterized by the assemblage of cells consisting of at least about 100,  
10 preferably about 1000, particularly preferably about 10000, pigment epithelial cells, and the latter not being separable from one another by moderate shear forces, in particular by repeated, for example ten-fold, movement up and down in a solution using a  
15 pipette.

A further aspect of the present invention is a cultivation system comprising at least one pigment epithelial cell of the eye and a feeder layer. The  
20 growing of IPE and RPE cells after isolation thereof is usually very time-consuming. The cultivation system of the invention allows large numbers of IPE and RPE cells to be produced in a very short time.

25 A feeder layer is understood by the skilled worker to mean cells which are cocultivated with other cells (target cells) and have a beneficial effect on the growth of the target cells. A beneficial effect may mean, for example, a faster growth of the cells or  
30 prevention of differentiation or dedifferentiation. This takes place, for example, by the cells of the feeder layer secreting molecules into the medium which then have a beneficial effect on growth of the target cells.

35 Inactivated fibroblasts are normally used as feeder layer for cultivating embryonic stem cells. Inactivation of the fibroblasts can be achieved, for example, by treatment with mitomycin C or by exposure

to  $\gamma$  rays. It is possible, for example, to use fibroblasts from a mammal, in particular mouse or human. In one implement, the fibroblasts and pigment epithelial cells of the same species, in particular of  
5 the same individual, are used. However, the fibroblasts may also be a permanent cell line, for example STO fibroblasts or 3T3 fibroblasts, or primary embryonic fibroblasts. The production of fibroblasts is known to the skilled worker (e.g. Abbondanzo S, Gadi I, Stewart  
10 C (1993) Derivation of embryonic stem cell lines. Methods in Enzymology 225: 803-823).

The cultivation system might, for example, comprise a culture vessel in which the pigment epithelial cells of  
15 the eye and the feeder layer are cultivated directly adjacent to one another, in particular one on top of the other, in a suitable medium. The different cells [lacuna], however, also be cultivated in a culture vessel spatially separate from one another so that the  
20 exchange of, for example, factors to stimulate growth takes place solely through the medium.

A further aspect of the present invention is a method for the production of a pigment epithelial cell of the  
25 eye, where the cell is genetically modified with the aid of an adenoviral vector of large DNA capacity.

A genetic modification is understood by the skilled worker to be any alteration of the genetic information  
30 of the cells. This can be achieved, for example, by addition, insertion, substitution and/or deletion of one or more nucleotides. In a particular embodiment, the genetic modification is brought about by gene transfer, it being possible for the gene to be, for  
35 example, present extrachromosomally in the cell.

Gene transfer means the introduction of one or more genes into, for example, a cell. In the present invention it is possible, for example, for at least one

gene to be introduced with the aid of an adenoviral vector with large DNA capacity into a pigment epithelial cell. cDNAs are normally used. However, it is also possible to use the genes themselves (including  
5 their introns and exons). In another embodiment, however, it is also possible to introduce a genetically modified, naturally occurring gene or synthetic nucleic acids into the pigment epithelial cell.

10 A further aspect of the present invention is a method for the production of a pigment epithelial cell of the eye comprising an adenoviral vector of large DNA capacity, where the cell is cultivated in serum-free medium, in the presence of a feeder layer and/or in a  
15 fixed assemblage of cells.

To isolate pigment epithelial cells in a fixed assemblage of cells, the iris or a part of the iris, or the retina, in particular in the peripheral retinal  
20 region, is separated, for example mechanically or enzymatically, in particular with Accutase, chondroitinase and/or heparinase, in particular from the stroma and the basal membrane. The cell sheet can then be cultivated further in cell culture. If  
25 required, the cell sheets can be broken down into single cells by renewed incubation with Accutase.

It has been possible to show within the scope of the present invention that a cell culture medium which  
30 contains no serum, for example fetal calf serum, has a beneficial effect on the growth of pigment epithelial cells of the eye.

A further aspect of the present invention is the use of  
35 genetically modified pigment epithelial cells for the therapy of eye diseases, possibilities being both a hereditary and an acquired eye disease. Examples of acquired or hereditary eye diseases are age-related

macular degeneration, glaucoma and diabetic retinopathy.

Age-related macular degeneration (AMD) is the commonest  
5 reason for legal blindness in western countries. Atrophy of the submacular retinal pigment epithelium and the development of choroidal neovascularizations (CNV) results secondarily in loss of central visual  
10 acuity. For the majority of patients with subfoveal CNV and geographic atrophy there is at present no treatment available to prevent loss of central visual acuity. Early signs of AMD are deposits (druses) between retinal pigment epithelium and Bruch's membrane. During  
15 the disease there is sprouting of choroid vessels into the subretinal space of the macula. This leads to loss of central vision and reading ability. An example of a therapeutic gene which can be employed for therapy of AMD is the PEDF gene.

20 Glaucoma is the name given to a group of diseases in which the pressure in the eye increases abnormally. This leads to restrictions of the visual field and to the general diminution in the ability to see. The commonest form is primary glaucoma; two forms of this  
25 are distinguished: chronic obtuse-angle glaucoma and acute angle closure. Secondary glaucoma may be caused by infections, tumors or injuries. A third type, hereditary glaucoma, is usually derived from developmental disturbances during pregnancy. The  
30 aqueous humor in the eyeball is under a certain pressure which is necessary for the optical properties of the eye. This intraocular pressure is normally 15 to 20 millimeters of mercury and is controlled by the equilibrium between aqueous production and aqueous  
35 outflow. In glaucoma, the outflow of the aqueous humor in the angle of the anterior chamber is blocked so that the pressure inside the eye rises. Glaucoma usually develops in middle or advanced age, but hereditary forms and diseases are not uncommon in children and

adolescents. Although the intraocular pressure is only slightly raised and there are moreover no evident symptoms, gradual damage occurs, especially restriction of the visual field. Acute angle closure by contrast  
5 causes pain, redness, dilation of the pupils and severe disturbances of vision. The cornea becomes cloudy, and the intraocular pressure is greatly increased. As the disease progresses, the visual field becomes increasingly narrower, which can easily be detected  
10 using a perimeter, an ophthalmological instrument. Chronic glaucoma generally responds well to locally administered medicaments which enhance aqueous outflow. Systemic active substances are sometimes given to reduce aqueous production. However, medicinal treatment  
15 is not always successful. If medicinal therapy fails, laser therapy or conventional operations are used in order to create a new outflow for the aqueous humor. Acute glaucoma is a medical emergency. If the intraocular pressure is not reduced within 24 hours,  
20 permanent damage occurs.

A numbers of growth or neurotrophic factors are able to withstand the survival of glaucomatous neurons. These include NGF, BDNF, CNTF, bFGF and neurotrophin 3,4-5.  
25 It would be possible in a preferred embodiment to use genetically modified pigment epithelial cells which contain as therapeutic gene the gene for NGF, BDNF, CNTF, bFGF and/or neurotrophin 3,4-5 for the therapy of glaucoma. These factors would then be able to regulate  
30 the survival by activating specific metabolic pathways. Many of these factors have a short half-life. Stable expression of these factors is accordingly of considerable therapeutic importance.

35 Diabetic retinopathy arises in cases of diabetes mellitus [lacuna] thickening of the basal membrane of the vascular endothelial cells as a result of glycosilation of proteins. It is the cause of early vascular sclerosis and the formation of capillary

aneurysms. These vascular changes lead over the course of years to diabetic retinopathy. The vascular changes cause hypoperfusion of capillary regions. This leads to lipoid deposits (hard exudates) and to vasoproliferation. The clinical course is variable in patients with diabetes mellitus. In age-related diabetes (type II diabetes), capillary aneurysms appear first. Thereafter, because of the impaired capillary perfusion, hard and soft exudates and dot-like hemorrhages in the retinal parenchyma appear. In later stages of diabetic retinopathy, the fatty deposits are arranged like a corona around the macula (retinitis circinata). These changes are frequently accompanied by edema at the posterior pole of the eye. If the edema involves the macula there is an acute serious deterioration in vision. The main problem in type I diabetes is the vascular proliferation in the region of the fundus of the eye. The standard therapy is laser coagulation of the affected regions of the fundus of the eye. The laser coagulation is initially performed focally in the affected areas of the retina. If the exudates persist, the area of laser coagulation is extended. The center of the retina with the site of sharpest vision, that is to say the macula and the papillomacular bundle, cannot be coagulated because the procedure would result in destruction of the parts of the retina which are most important for vision. If proliferation has already occurred, it is often necessary for the foci to be very densely pressed on the basis of the proliferation. This entails destruction of areas of the retina. The result is a corresponding loss of visual field. In type I diabetes, laser coagulation in good time is often the only chance of saving patients from blindness.

35

One example of a genetically related disease of the pigment epithelium is autosomal recessive severe retinal dystrophy which starts in childhood and is caused by mutation in the RPE65 gene (Gu SM, Thompson

DA, Srikumari CR, Lorenz B, Finckh U, Nicoletti A, Murthy KR, Rathmann M, Kumaramanickavel G, Denton MJ, Gal A (1997) Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy. Nat Genet 17: 194-197. Correction of the pathological phenotype is to be expected from introduction of the RPE65 gene with the aid of an adenoviral vector with large DNA capacity.

10 It was additionally completely surprising that pigment epithelial cells of the eye can also be transplanted into the CNS. It was possible to show within the framework of the present invention that pigment epithelial cells survived the observation period of 5 weeks. Histological examination revealed no evidence of induction of damage to neural cells. Instead it was possible to observe that the pigment epithelial cells formed intensive contacts with neurons.

20 To date, a number of different cell types have been employed in animal experiments or in patients with Parkinson's disease in clinical studies:

Examples are fetal cells obtained from brains of human fetuses. Fetal cells from the ventral midbrain or dopaminergic neurons have already been transplanted in clinical studies on more than 300 patients with Parkinson's disease (for review, see Alexi T, Borlongan CV, Faull RL, Williams CE, Clark RG, Gluckman PD, Hughes PE (2000) (Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. Prog Neurobiol 60: 409-470). A number of different cell types, including non-neuronal cells, e.g. cells from the adrenal cortex, Sertoli cells on the gonads or glomus cells from the carotid bodies, fibroblasts or astrocytes, have been used in patients with Parkinson's disease or in animal models with the aim of replacing dopamine spontaneously or after gene transfer (Alexi et al. 2000, supra). The survival rate



of transplanted fetal dopaminergic neurons is 5-8%, which was enough to cause a slight improvement in the signs and symptoms (Alexi et al. 2000, supra).

- 5 In recent years, neuronal stem cells from brains of adult vertebrates have been isolated, expanded in vitro and reimplanted into the CNS, after which they differentiated into pure neurons. Their function in the CNS remains uncertain, however. Neuronal precursor  
10 cells have also been used for gene transfer (Raymon HK, Thode S, Zhou J, Friedman GC, Pardinas JR, Barrere C, Johnson RM, Sah DW (1999) Immortalized human dorsal root ganglion cells differentiate into neurons with nociceptive properties. J Neurosci 19: 5420-5428).
- 15 Schwann cells which overexpressed NGF and GDNF had neuroprotective effects in models of Parkinsonism (Wilby MJ, Sinclair SR, Muir EM, Zietlow R, Adcock KH, Horellou P, Rogers JH, Dunnett SB, Fawcett JW (1999) A glial cell line-derived neurotrophic factor-secreting  
20 clone of the Schwann cell line SCTM41 enhances survival and fiber outgrowth from embryonic nigral neurons grafted to the striatum and to the lesioned substantia nigra. J Neurosci 19: 2301-2312).
- 25 The advantage of pigment epithelial cells, compared with cells used to date, especially on use of endogenous (autologous) cells, is that they are not rejected by the immune system and thus, as expected, have a very high survival rate. In addition, they  
30 replace natural melanin pigment which is lost in the substantia nigra of Parkinsonian patients. This melanin is able to detoxify free  $Fe^{++}$  and thus has a beneficial effect on the progress of the disease.
- 35 Another aspect of the present invention is therefore the use of pigment epithelial cells for the therapy of nerve diseases, in particular a disease of the nervous system, preferably of the CNS, especially of Parkinson's disease.

An example of a common disease of the CNS is Parkinson's disease which is a chronic degenerative disease of the brain. The disease is caused by  
5 degeneration of specialized neuronal cells in the region of the basal ganglia. The death of dopaminergic neurons results in reduced synthesis of dopamine, an important neurotransmitter, in patients with Parkinson's disease. The standard therapy is medical  
10 therapy with L-dopa. L-Dopa is metabolized in the basal ganglia to dopamine and there takes over the function of the missing endogenous neurotransmitter. However, L-dopa therapy loses its activity after some years.

15 Pigment epithelial cells spontaneously produce some factors which have a neuroprotective effect. Examples of such factors are, which are produced, for example, by IPE cells, are nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), basic fibroblast growth  
20 factor (bFGF) or factors with an angiogenic activity such as, for example, vascular endothelial growth factor (VEGF) or platelet-derived growth factors A and B (PDGF A+B). An example of a neurotrophic factor which can be produced in the genetically modified IPE cells  
25 after gene transfer is glial cell-derived neurotrophic factor (GDNF).

It is additionally possible to utilize the natural protective function of pigment epithelial cells. In  
30 Parkinson's disease, transplanted IPE cells may display a neuroprotective effect due to the antioxidant effect of their melanin granules. This could be caused by the ability of melanin and its precursors to bind  $\text{Fe}^{2+}$  and other toxic substances (e.g.  $\text{Ca}^{2+}$ ) and thus remove them  
35 from the cell cytoplasm (Hill HZ (1992) The function of melanin or six blind people examine an elephant. Bioessays 14: 49-56) or to have a very strong antioxidant effect. IPE cells have a high melanin content and also continue to form melanin when they are

located in the retina, the subretinal space or the CNS. Unambiguous proof thereof is the presence of numerous early stages of melanogenesis (premelanosomes), detectable by electron microscopic studies. Melanin has  
5 antioxidant properties, protects from lipid peroxidation, is able directly to bind oxygen free radicals (Hill HZ (1992) The function of melanin or six blind people examine an elephant. Bioessays 14: 49-56) and can prevent the formation of new oxygen free  
10 radicals by binding metal cations (Memoli S, Napolitano A, d'Ischia M, Misuraca G, Palumbo A, Prota G (1997) Diffusible melanin-related metabolites are potent inhibitors of lipid peroxidation. Biochim Biophys Acta 1346: 61-68). If highly pigmented iris pigment  
15 epithelial cells are introduced into tissue with high oxidative stress, for example in the substantia nigra of patients with Parkinson's disease, or into the papilla of glaucoma patients, or into the vicinity of the macula of AMD patients, then a neuroprotective  
20 effect occurs simply through the presence of the melanin in the IPE cells.

An example of a protein with a good therapeutic potential for the therapy of patients with Parkinson's  
25 disease is glial cell-derived neurotrophic factor (GDNF), a survival factor for dopaminergic neurons. GDNF has effects even in picomolar concentrations on the survival rate and growth of dopaminergic neurons from embryonic brain. Animal experimental studies have  
30 shown that direct gene transfer into the substantia nigra of a GDNF expression cassette using various vectors (first generation adenoviral vectors, AAV vectors or lentiviral vectors) was able to protect dopaminergic neurons in the 6-OHDA rat model (Mandel  
35 RJ, Spratt SK, Snyder RO, Leff SE (1997) Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of

cells of the invention can display their therapeutic effect through transplantation into the eye.

5 The pigment epithelial cells can be transplanted, for example, into the choroid and exert there a therapeutic effect through production of pigment epithelial- endogenous factors or through production of therapeutic molecules after genetic modification with an adenoviral vector of large DNA capacity.

10

However, the pigment epithelial cells can also be employed in the region of the papilla. It has been possible to show within the scope of the present invention that, after injection into the posterior part  
15 of the vitreous, the cells migrate into the optic disk and there integrate into the tissue assemblage. This opens up the possibility of therapy of diseases manifested, for example, in the optic disk.

20 The pigment epithelial cells can also be injected into the vitreous, in particular through a transscleral access into the posterior part of the vitreous. It was possible to show, by fundoscopic inspection of RCS rats, that the IPE cells are found entirely on the  
25 papilla throughout an observation period of 2 months. It was histologically evident that the IPE cells had migrated into the optic disk and there formed intensive contact with the blood vessels and axons. Electronic microscopy shows no damage to neuronal cells or  
30 proliferation of IPE cells in the vitreous. This embodiment of the invention makes it possible, for example, to have direct access to the optic disk and to release or activate, with or without genetic modification, neuroprotective mediators in or in the  
35 direct vicinity of the papilla.

A further embodiment of the present invention relates to the use of adenoviral vectors with large DNA capacity for the genetic modification of pigment

epithelial cells in vivo. As shown in the examples, in vivo transduction of RPE cells leads, through subretinal injection with an adenoviral vector of large DNA capacity, to a surprisingly stable expression for  
5 at least 6 months.

A further aspect of the present invention relates to a medicament or diagnostic aid comprising a pigment epithelial cell of the invention and suitable  
10 excipients and/or additives. Suitable excipients and additives, which serve, for example, to stabilize or preserve the medicament or diagnostic aid, are generally known to the skilled worker (see, for example, Sucker H et al. (1991) Pharmazeutische  
15 Technologie, 2<sup>nd</sup> edition, Georg Thieme Verlag, Stuttgart). Examples of such excipients and/or additives are physiological saline solutions, Ringer dextrose, dextrose, Ringer lactate, demineralized water, stabilizers, antioxidants, complexing agents,  
20 antimicrobial compounds, proteinase inhibitors and/or inert gases.

The following figure and the examples are intended to explain the invention further without restricting it  
25 thereto.

#### DESCRIPTION OF THE FIGURE

Fig. 1 Schematic cross section through the right eye.  
30

The iris pigment epithelium is located on the side of the iris facing the lens. The macula is the region (about 6 mm in diameter) directly surrounding the fovea.

35

#### EXAMPLES

##### 1. Isolation of cell sheets

To isolate IPE cells in assemblages of cells (cell sheets), iridectomies were collected fresh from the operating theater after a trabeculectomy or a basal iridectomy, brought in F12 medium ((HAM) with L-glutamine, Gibco, Life Technologies, Paisely, Scotland) and directly processed further. Basal iridectomies of glaucoma patients or pieces of iris from rats or pigs were treated with Accutase (Cat. No. L11-007, PAA Laboratories) in Dulbecco's PBS with 0.5 mmol/l EDTA x Na for 15-20 min. The tissue which can be obtained by an iridectomy has an area of about 3.5 mm<sup>2</sup> and contains about 20000 IPE cells. The cell layers were pipetted up and down very carefully with F12 medium and pipetted out on polystyrene. It was possible to detach the IPE cells completely as double cell layer with intact basal membrane from the stroma under the stereomicroscope as was demonstrable by examination under the electron microscope. It was possible to remove this basal membrane completely by incubation with 0.1 U/ml chondroitinase ABC (Sigma) and 2.4 U/ml heparinase (Sigma) in PBS at pH 7.4 and at 37 degrees Celsius for 2 hours. It was then possible to break the cell sheets down into single cells by renewed Accutase incubation for 5 minutes.

To isolate RPE cells in assemblages of cells (cell sheets), autologous RPE cell sheets and single cells were mechanically detached in the periphery of human eyes after local retinotomy, and aspirated with a canula. It was possible to remove 50,000 peripheral RPE cells locally without the patients later complaining of unpleasant serious losses of visual field. The removal of 50,000 RPE cells in each case was possible at several peripheral sites on the eye.

## 2. Growing of IPE and RPE cells by cultivation on fibroblasts

- 5 IPE or RPE cells obtained from iridectomies or eyes of organ donors were cultivated on fibroblasts (mouse 3T3 fibroblast cell line), which served as feeder layer, in F12 medium. The cells become adherent to the fibroblasts within one day and start to proliferate.
- 10 The number of cells tripled or quadrupled on the fibroblasts within 3 days. The fibroblasts had previously been treated with 40 µg/ml mitomycin C so that they die after no more than 10 days. A pure culture of pigment epithelial cells is obtained after
- 15 this time.

## 3. Injection of IPE cells

- IPE cells were isolated as single cell suspension as described under 1. An animal model of age-dependent macula degeneration and of retinal degeneration caused by a specific phagocytosis defect and degeneration of the RPE is the Royal College of Surgeons Rat (RCS rat). Under Ketanest/Nembutal anesthesia, the upper
- 20 conjunctiva of dystrophic RCS rats (18 days old) was opened by an incision 4 mm long and 4 mm posterior of the limbus. Kolibri forceps were used to hold the conjunctiva near the limbus firmly.

### 3.1. Subretinal injection

- In the subretinal injection, a 26 gage canula was used to pierce the sclera, choroid and retinal pigment epithelium at the level of the equator as far as the vitreous. A Hamilton syringe with a blunt 32 gage
- 35 canula was introduced anteriorly 2-3 mm tangentially between retina and RPE. 60000 IPE cells were injected in 0.5 µl of cell culture medium (F 12 (HAM) with L-glutamine, Gibco, Life Technologies, Paisely Scotland). The RCS rats were sacrificed after completion of the

observation period of 6 and 8 months. The eyes were enucleated. The corneas were removed and the remaining parts of the eyes were fixed in 3% glutaraldehyde. Areas with transplanted iris cells were easily  
5 identifiable from the pigmentation and were excised and embedded for electron microscopic investigations in accordance with a routine protocol. Under the electron microscope, surviving, i.e. morphologically intact, IPE cells were detectable in the subretinal space for up to  
10 8 months after transplantation. Surviving photoreceptors with inner segments but without outer segments were present for up to 6 months after subretinal transplantation.

### 15 3.2. Injection into the vitreous

Injection into the vitreous took place at the same site of the subretinal injection in 6 eyes. However, the canula was introduced like a secant of a circular arc 1-2 mm deep into the vitreous. 60000 IPE cells were  
20 injected close to the papilla. The vitreous and the lens remained clear in the observation period of 2 months. The IPE cells in all 6 eyes formed a macroscopically or funduscopically visible collection on the papilla. The histology showed that the IPE cells  
25 migrated into the optic disk. The cells were highly pigmented and there was no evidence of cell damage or proliferation.

### 3.3. Injection into the choroid

30 The site chosen for injection into the choroid was the same as for subretinal injection. The sclera was cut with a pointed scalpel through an incision 1 mm long until the choroid was visible. The canula was placed perpendicular to the eyeball on the incision site and  
35 60000 IPE cells in 0.5  $\mu$ l of F12 medium were injected into the choroid. IPE cells transplanted into the choroid in 15 eyes, compared with 6 untreated eyes, led to a survival of photoreceptors for up to 6 months. Both the number of surviving photoreceptors/mm of



retina ( $p=0.020$ ) and the maximum nucleus height ( $p=0.019$ ) were significantly different in the Mann-Whitney test from the untreated eyes (Table 1).

	Number of eyes	Photo- receptor nuclei [mm <sup>-1</sup> ]	Maximum height of the photoreceptor cell nuclei
<u>IPE transplantation</u>	15		
Median		12.3	1.0
25 <sup>th</sup> Percentile		0.0	0.0
75 <sup>th</sup> Percentile		45.5	3.0
<u>Control without treatment</u>	6		
Median		0.0	0.0
25 <sup>th</sup> Percentile		0.0	0.0
75 <sup>th</sup> Percentile		0.0	0.0
<u>Mann-Whitney test</u>			
P value		<u>0.020</u>	<u>0.019</u>

5

Table 1: Median, 25<sup>th</sup> and 75<sup>th</sup> percentiles of the number of photoreceptor cell nuclei still present, of the maximum thickness of the photoreceptor layer in semithin sections 6 months after transplantation of IPE cells into the choroid is indicated.

10

#### 4. Injection of rat IPE cells into the CNS

For the stereotactic implantation method, Wistar rats were anesthetized by intraperitoneal injection of 1 ml of avertin (2 g of tribromoethanol 3,3,3 (dry), 1 g of pentanol (liquid), 8 ml of 100% ethanol and 90 ml of 0.9% NaCl) per 100 g of body weight. The cranium was fixed in a precisely reproducible manner at three bone points, the external auditory canals and the maxilla so that the calvaria was horizontal at the level of the bregma. After the medial front-occipital skin incision, 1.5 cm long, the periosteum was pushed aside in order

15

20

to have a clear view of the cranium sutures which served as reference point for the stereotactic coordinates. The coordinates were determined on the basis of the atlas of Praxinos and Watson (Praxinos G. Watson C. The rat brain in stereotactic coordinates. 1986;2 end Dr., Academic Press, Sydney):

The puncture site was 1.5 mm in the frontal direction and 2 mm to the right parietally from the bregma. The upper portion of the striatum is at a depth of 4.5 mm. The hole with a diameter of about 0.5 mm was drilled at the appropriate position using a precision shaft drill (Proxxon, Minimoto 40IE) avoiding damage to the dura. 5-10  $\mu$ l of the cell suspension were introduced through this drilled hole with a 25  $\mu$ l N-702-N Hamilton syringe with fixed needle, injecting at a depth of 5 mm measured from the surface of the dura. 60000 IPE cells from Long Evans rats were injected into the striata of each of 4 Wistar rats. Before the needle was withdrawn, 2 min were allowed to elapse so that the cell suspension was able to diffuse into the tissue and the resulting local pressure could diminish. Otherwise there was a risk that cells could have followed the withdrawn needle into the puncture channel or into the overlying tissue sections. For the same reason, a further 30 sec were allowed to elapse after the needle had been withdrawn 4 mm, before it was completely removed. A skin suture was then applied.

After 5 weeks, the brains were perfusion-fixed with 3% glutaraldehyde in cacodylate buffer. Pigmented areas were excised from the striatum and embedded for electron microscopy.

The transplanted areas were easily identifiable macroscopically by the pigmentation. Under the electron microscope, the IPE cells had intact mitochondria and plasma membranes. They were highly pigmented, contained melanogenesis stages and formed contact zones with

neurons. The IPE cells were always found singly without contacts with other IPE cells. They were also found 3-4 mm away from the puncture channel, which suggests active migration of the cells. The neurons adjacent to the IPE cells were morphologically intact. Immunocompetent cells (macrophages, lymphocytes) were not observed.

### 5. Genetic modification

Isolated and adherent rat and human IPE cells were transduced in vitro with 20, 50 and 100 MOI (multiplicity of infection) of the adenoviral vector with large DNA capacity HC adenovirus "HC-AdFK7" which harbors the EGFP (enhanced green fluorescent protein) under the control of the human CMV (cytomegalovirus) promoter as transgene. For this purpose, 80% confluent cell cultures in F12 complete medium were incubated with the appropriately diluted virus stock solution at 37°C for 24 hours. The medium was changed the next morning. Expression of the transgene was checked as green fluorescence inside the cells under a fluorescence microscope with FITC filter [lacuna] 24, 48 and 72 hours and then each subsequent week.

As soon as 24 hours after the transduction a slight fluorescence was visible and became distinctly stronger in subsequent days. The human IPE cells were 100% transduced with 100 MOI, and the rat cells were 80% transduced with 20 MOI and 100% transduced with 50 and 100 MOI. Expression was detectable in vitro for a period of up to 8 weeks or longer.

In a further experiment, the adenoviral vector of large DNA capacity AdhCMV.PEDF was constructed. This vector expresses the human PEDF cDNA under the control of the human CMV promoter. The PEDF protein is additionally tagged by a poly-histidine epitope expressed as fusion protein with the PEDF. This vector was produced by a

standard method (Schiedner G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C, Graham FL, Beaudet AL, Kochanek S (1998) Genomic DNA transfer with a high capacity adenovirus vector results in improved in vivo  
5 gene expression and decreased toxicity. Nature Genetics 18: 180-183) in Cre-recombinase-expressing 293 cells and purified by CsCl density gradient centrifugation.

Cytokeratin-positive human IPE cells from the 2<sup>nd</sup>  
10 passage were transduced with the HC-Ad.CMV.PEDF vector. For this purpose, 80% confluent cell cultures in F12 complete medium were incubated with the appropriately diluted virus stock solution at 37°C for 24 hours. The medium was changed next morning. Expression of the  
15 transgene and secretion of the PEDF into the culture supernatant were checked in an ELISA using specific anti-polyhistidine antibodies after 72 hours. The culture supernatants contained 150 ng PEDF/ml. This corresponds to a production of 60 pg of PEDF per 1000  
20 cells in 72 hours. It was possible to detect in another ELISA using another antibody which specifically recognizes the human PEDF protein that human IPE cells do not, in contrast to human RPE cells, spontaneously produce PEDF.

25

#### *IPE sheet transfection*

It was possible to transfect not only single cells but also cell sheets. IPE cell sheets could be transfected after enzymatic removal of the basal membranes. The  
30 cell sheets with intact basal membrane could not be transfected, as was demonstrated by PCR.

For transfection of cell sheets, pig eyes were brought to the laboratory, and processed further, from the  
35 slaughterhouse immediately after the animals were slaughtered. The anterior segment was removed by a circular cut about 2 mm behind the limbus. The iris was then removed by a blunt dissection from the posterior direction and incubated in 1 ml Accutase at 37°C for 15

min. The IPE is then detached from the stroma of the iris using a glass pipette bent in a flame. Estimation of the area of the individual IPE cell sheets obtained resulted in between 40,000 and 70,000 IPE cells per  
5 cell sheet.

The IPE sheets were incubated with 200 MOI of the EGFP-expressing adenoviral vector HC-AdFK7 for 24 h. The medium was changed after 24 hours. The IPE sheets were  
10 cultivated in F12 complete medium for 6 days and investigated for EGFP fluorescence, but it was not possible to demonstrate this with certainty because of the morphology of the cells with very densely concentrated melanin granules. DNA was then obtained  
15 from the cells using the QIAmp DNA mini kit (Qiagen). The manufacturer's instructions were followed. The transgene was detected by PCR using the primers prod1, which binds in the region of the CMV promoter, and prod2, which binds in the region of the EGFP sequence,  
20 to the DNA of HC-AdFK7. With successfully transduced cells, prod1 and prod2 produced a PCR product with a length of about 700 base pairs. A plasmid pFK7 with the same insert as is to be found in HCAdFK7 served as positive control.

25

#### 6. Subretinal transplantation of genetically modified cells

8 Wistar rats received subretinal transplantation, in 8  
30 eyes, of IPE cells, transduced with HC-AdFK7 vector, from Long Evans rats (albinotic) by the same method as described for untransfected IPE cells.

Four eyes into which IPE cells had been transplanted  
35 were enucleated after 2 months, embedded in tissue freezing medium (Jung, Heidelberg, Germany) and frozen at -80°C. Cryostat sections (7 µm) were, after thawing, embedded in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany) and examined under a Zeiss Axiophot

light microscope with an excitation wavelength of 400-400 nm and an emission wavelength of 470 nm. The subretinally transplanted IPE cells showed distinct expression of green fluorescent proteins.

5

For evaluation using the scanning laser ophthalmoscope (SL), the other 4 transfected rats were examined 14 days and 4 months after the transfection. The animals were anesthetized with Ketanest and evaluated using the scanning laser ophthalmoscope (Rodentstock, Munich). This entailed scanning of the retina of the rats with mydriasis in fluo mode with the infrared laser (780 nm), the argon green laser (514 nm) and argon blue laser (488 nm). In this mode, the instrument uses a fluorescein cutoff filter suitable for observing the EGFP fluorescence. The images were recorded by S-VHS video. The analog video images were copied digitally onto DV, and bitmaps were produced from representative sections and evaluated for area and intensity using the software Optimas 6.1.

During the observation period of 4 months, the intensity of fluorescence caused by the transfected IPE cells, and the extent of the transplanted areas in the fundus remained constant in all 4 eyes, i.e. expression of the transfected genes remained unchanged at the protein level.

#### 7. Subretinal injection of free vector for genetic modification of RPE cells of the host (in vivo gene therapy)

For subretinal injection of free vector in vivo, various concentrations of an HC adenovirus "HC-AdFK7" which harbors the EGFP (enhanced green fluorescent protein) gene under the control of a CMV (cytomegalovirus) promoter as transgene were injected subretinally into Wistar rats. Expression of the transgene were evaluated using the scanning laser

ophthalmoscope (Rodenstock, Munich). This entailed scanning of the retina of the rats with mydriasis in fluo mode with an infrared laser (780 nm), the argon green laser (514 nm) and argon blue laser (488 nm). In this mode, the instrument uses a fluorescein cutoff filter suitable for observing the EGFP fluorescence. The images were recorded by S-VHS video. The analog video images were copied digitally onto DV, and bitmaps were produced from representative sections and evaluated for area and intensity using the software Optimas 6.1.

During the observation period of 6 months, the intensity of the fluorescence caused by the transfected IPE cells, and the extent of the transplanted areas in the fundus remained constant in all 4 eyes, i.e. expression of the transfected genes remained unchanged at the protein level. After 6 months, the animals were sacrificed, and the eyes were fixed in 3% glutaraldehyde. The anterior segments of the eyes were removed and the remaining posterior optic cups were divided into four. After the retinas had been removed, the sclera, choroid with pigment epithelium were examined under a fluorescence microscope (Axiovert Zeiss, Oberkochen, Germany) using a 450-490 nm excitation filter and a 520 nm emission filter (AF Analysentechnik, Tübingen, Germany). This revealed the typically hexagonal shape of transduced and EGFP-positive pigment epithelial cells.

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#### 8. Transplantation of genetically modified cells into the CNS

For transplantation of genetically modified cells into the CNS, IPE cells (60000) which had been transduced with HC-AdFK7 vector and expressed EGFP from Long Evans rats were stereotactically injected as described above into the striatum of each of 4 Wistar rats.

After 8 weeks, the animals were sacrificed by cervical dislocation under CO<sub>2</sub> anesthesia. The brains were dissected out. Pigmented areas with transplanted cells were excised from the striatum and frozen in tissue freezing medium (Jung, Heidelber, Germany). The fluorescence caused by the expression of EGFP by the IPE cells was detectable in frozen sections 8 weeks after transplantation in pigmented cells.

10 9. Prevention of choroidal neovascularization [lacuna]  
genetically modified and PEDF-expressing IPE cells in  
vivo

IPE cells were cotransfected with the adenoviral vector of large DNA capacity HC-AdFK7, which harbors the EGFP (enhanced green fluorescent protein) gene under the control of a CMV (cytomegalovirus) promoter as reporter gene, and simultaneously with the PEDF-expressing adenoviral vector of large DNA capacity HC-AdCMV.PEDF in vitro and, after 6 days, transplanted into the subretinal space of Long Evans rats (60,000 cells/eye) (1<sup>st</sup> experimental group). The PEDF expression cassette in the adenoviral vector of large DNA capacity contained a poly-HIS epitope for detecting the protein using an anti-HIS antibody in addition to the PEDF-encoding sequence. One week after injection, the rats were anesthetized, the pupils were dilated and the rats received 3-4 laser burns around the optic nerve with a blue-green argon laser (Coherent, Inc., Santa Clara, CA, USA). The energy of the laser was 90 mW for 100 ms, and the diameter of the beam was 100 µm. A second group of rats received only laser burns without genetic modification by cell transplantation or free vectors. After 16 days, the rats were anesthetized and received 0.5 ml Liquemin i.p. (Roche, Grenzach-Wyhlen, Germany). The ascending aorta was cannulated and, after the right atrium was opened, the blood was washed out with 50 ml of Ringer lactate solution (Stereofundin, Braun, Melsungen, Germany). This was followed by perfusion



with 20 ml of Ringer solution with 5 mg/ml FITC dextran (Sigma Deisenhofen, Germany). The eyes were enucleated, pierced with a scalpel at the level of the limbus and fixed in 4% paraformaldehyde overnight. The next day, the anterior segment of the eyes was removed up to a short distance behind the ora serrata by a circular incision. The remaining optic cup was divided into quadrants by 4 radial incisions, and the retinas were removed. Quadrants consisting of pigment epithelium, choroid and sclera containing laser scars were incubated in tris buffer (TBS) for 4 x 10 min and then in 0.5 M NH<sub>4</sub>CL (Sigma, Deisenhofen, Germany) and 0.25% Triton (Serva, Heidelberg, Germany) for 10 min. After two further washes, the specimens were incubated with 5% BSA (Albumin, Bovine Fraction Sigma, Deisenhofen, Germany). Some of the specimens were incubated with antibodies against histidine (anti-His antibody, Qiagen, Hilden, Germany) in order to detect the histidine residues in the PEDF. The primary antibodies were visualized using anti-mouse IgG coupled to the fluorescent dye Cy3 (Rockland, Gilbertsville, PA, USA).

Other specimens were treated with rat anti-mouse CD 31 (PECAM-1, Pharmingen, San Jose, CA, USA) in order to visualize the endothelial cells. This was followed by a 2<sup>nd</sup> incubation with anti-rat IgG-biotin (Amersham, Pharmacia Biotech Europe GmbH, Freiburg, Germany) with subsequent localization of the biotin by Fluorolink Cy3 (Amersham Life Sciences, Braunschweig, Germany). Some choroid specimens underwent double labeling of PEDF and endothelial cells. In these cases, PEDF expression was visualized with Cy3 as described, and the PECAM-biotin complex was visualized with streptavidin-Alexa Fluor 350 (MoBiTec, Göttingen, Germany).

The flatmount specimens were evaluated under the fluorescence microscope (Axiophot, Zeiss, Oberkochen, Germany).

In group 1 there was no observable escape of FITC-dextran nor an increased occurrence of CD 31-positive cells in 16 of 19 laser scars when PEDF-expressing IPE cells were present at a distance of 100-1000  $\mu\text{m}$  from the scar. The expression of PEDF by the transplanted IPE cells was demonstrated with anti-His antibodies.

In the control group 2 (only laser burn), neovascularization was present in 9 of 12 laser scars. This was evident from the escape of dextran-FITC in and around the scar region and from the presence of flattened CD 31-positive endothelial cells in and around the scar region.

Neovascularization was thus detected in the same eye with a functional (dextran leakage) and an immunological method (direct detection of the newly produced endothelial cells with antibodies). These flatmount specimens allow the entire choroid to be assessed. These results show that neovascularization is inhibited by transplantation of IPE cells which express PEDF of an adenoviral vector of large DNA capacity.

# **ABSTRACT**

5 The present invention relates to a pigment epithelial cell of the eye containing vector DNA of an adenoviral vector with large DNA capacity, to the improved isolation and cultivation of these cells and to methods for production and the use in the therapy of an eye or nerve disease.

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Patent claims

1. Pigment epithelial cell of the eye, characterized  
in that the cell comprises vector DNA of an  
adenoviral vector with large DNA capacity.  
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2. Pigment epithelial cell of the eye according to  
claim 1, which is a retinal pigment epithelial  
cell or an iris pigment epithelial cell.  
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3. Pigment epithelial cell of the eye according to  
claim 1, where the vector DNA comprises at least  
one therapeutic nucleic acid, in particular a  
therapeutic gene, preferably for a neurotrophic  
factor such as GDNF, PEDF, NGF, BDNF, CNTF, bFGF  
or neurotrophin 3,4-5, an antiangiogenetic factor  
such as a soluble VEGF receptor-1 (sflt-1), a  
dominant-negative VEGF receptor-2 (KDR) or PEDF,  
an antioxidative factor such as superoxide  
dismutase, catalase or various peroxydases, a  
lysosomal factor such as alpha-mannosidase, beta-  
galactosidase, N-acetyl-beta-glucosaminidase, N-  
acetyl-beta-galactosaminidase, and lipase, or a  
vasodilating factor such as NO synthase.  
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25  
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4. Pigment epithelial cell of the eye according to  
claim 1, where the vector DNA comprises a  
constitutively active, regulatable and/or a  
tissue-specific promoter and/or a regulatable  
expression system.  
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5. Pigment epithelial cell of the eye according to  
claim 1, where the cell produces at least one  
therapeutic protein and/or a therapeutic RNA.

6. Pigment epithelial cell according to claim 1, where the cell is in a fixed assemblage of cells and/or has been cultivated in the presence of a feeder layer and/or in serum-free medium.
- 5 7. Pigment epithelial cell of the eye in the form of a fixed assemblage of cells.
8. Cultivation system comprising at least one pigment  
10 epithelial cell of the eye and a feeder layer.
9. Method for producing a pigment epithelial cell of the eye according to claim 1, characterized in that the cell is genetically modified with the aid  
15 of an adenoviral vector with large DNA capacity.
10. Method for producing a pigment epithelial cell of the eye according to claim 1, characterized in that the cell is cultivated in serum-free medium  
20 and/or in the presence of a feeder layer.
11. Method for producing pigment epithelial cells of the eye in the form of a fixed assemblage of cells according to claim 7, characterized in that the  
25 assemblage of cells is separated, in particular enzymatically, from surrounding tissue.
12. Method for producing pigment epithelial cells, characterized in that the cells are cultivated in  
30 a cultivation system according to claim 8.
13. Use of a pigment epithelial cell according to claim 1 in therapy of an eye disease, in particular of AMD, a glaucoma, diabetic  
35 retinopathy or a genetic disease of the pigment epithelium.
14. Use of a pigment epithelial cell according to claim 7 in therapy of an eye disease, in

particular of AMD, a glaucoma, diabetic retinopathy or a genetic disease of the pigment epithelium.

- 5 15. Use of a pigment epithelial cell according to claim 13 or 14, where the pigment epithelial cell is transplanted into the eye, in particular the choroid, into the papilla and/or into the vitreous.
- 10 16. Use of a pigment epithelial cell in therapy of a nerve disease, in particular a disease of the nervous system, preferably of the CNS, especially of Parkinson's disease.
- 15 17. Use of a pigment epithelial cell according to claim 1 in therapy of a nerve disease, in particular a disease of the nervous system, preferably of the CNS, especially of Parkinson's
- 20 disease.
18. Use of a pigment epithelial cell according to claim 16 or 17, where the pigment epithelial cell is transplanted into the nervous system, in
- 25 particular the CNS.
19. Use of a pigment epithelial cell according to claim 13 or 14, characterized in that the pigment epithelial cell is an autologous pigment
- 30 epithelial cell.
20. Use of a pigment epithelial cell according to claim 16, characterized in that the pigment epithelial cell is an autologous pigment
- 35 epithelial cell.
21. Medicament or diagnostic aid comprising a pigment epithelial cell of the eye according to claim 1 and other excipients and/or additives.